

Elevation of rat plasma P-selectin in acute lung injury

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Abstract

Acute lung injury in the rat caused by intravenous (i.v.) infusion of cobra venom factor (CVF) or lipopolysaccharide (LPS) is mediated by P-selectin-dependent neutrophil infiltration into the lung. In these lung injury models, P-selectin expression is induced on lung vascular endothelial cells after the CVF or LPS infusion, suggesting soluble P-selectin derived from inflamed sites might also be elevated. Here we established a sensitive enzyme-linked immunosorbent assay (ELISA) to measure soluble P-selectin in plasma, a potential marker of lung injury. Nine anti-rat P-selectin monoclonal antibodies that we established previously were first classified into 5 groups based on real-time biospecific interaction analyses, and used to develop a sandwich ELISA for accurately measuring the amount of soluble P-selectin in plasma. We then used this ELISA to measure the plasma P-selectin levels in Long Evans, Wistar, and Sprague–Dawley rats after the i.v. infusion of CVF or LPS. The elevation in P-selectin levels was significantly different among the strains, but it consistently correlated with the extent of lung inflammation, measured by myeloperoxidase levels in the lung tissues. Thus, our results indicate that the soluble P-selectin in plasma could serve as a sensitive biomarker reflecting lung inflammation, which is of clinical importance for detecting and preventing severe lung injury.

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1. Introduction

P-selectin (CD62P) is a member of the selectin family and is localized to platelet granule membranes and the Weibel–Palade bodies of endothelial cells. It is expressed on the cell surface upon cell activation and is the first point of contact for leukocytes adhering to activated platelets in thrombi or to activated endothelial cells, where it enables leukocyte rolling, thereby initiating the adhesion cascade.

Soluble P-selectin lacking the cytosolic/transmembrane domain has been identified in plasma. It is derived both from the secretion of an alternatively spliced protein that lacks the transmembrane domain and from proteolytic cleavage of the membrane form; thus, it reflects the activation state of platelets

and/or endothelial cells. The soluble form of P-selectin is detected as a circulating protein in plasma from healthy individuals [1,2], and it is an established marker of platelet activation [3,4]. High levels of this circulating protein are associated with several inflammatory and thrombotic disorders in humans, including diabetes [5], malaria [6], hepatitis [7], hypertension [8], atherosclerosis, and ischemic heart disease [9–11].

The expression of P-selectin on endothelial cells of the lung is up-regulated in rat models of lung injury induced by cobra venom factor (CVF) [12] or lipopolysaccharides (LPS) [13]. However, how much the soluble P-selectin level in plasma increases during lung injury remains unknown.

In the present study, we developed a sensitive P-selectin ELISA, and used it to measure the soluble P-selectin levels in the plasma of rats with acute lung injury induced by CVF or LPS. Our results indicate that soluble P-selectin could serve as a sensitive biomarker to measure the extent of lung injury.

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2. Materials and methods

2.1. Antibodies

The rat P-selectin-human IgG₁ fusion protein (P-selectin-IgG) and the hamster monoclonal antibodies (mAbs) 118, 136, 137, A109, A116, A123, A216, C104, and C215 against rat P-selectin were previously described [14]. The polyclonal antibody against rat P-selectin was obtained from BD Biosciences Pharmingen.

2.2. Immunoprecipitation analysis

Thrombin-activated rat platelets were prepared as described previously [14]. Rat platelets were activated with 3 U/ml thrombin (Sigma-Aldrich, St. Louis, MO, USA) for 10 min at 37 °C, then washed with PBS and surface-labeled with 5 ml of 50 µg/ml sulfo-NHS-LC-biotin (Pierce Biotechnology, Inc., Rockford, IL, USA) for 60 min at 4 °C. The platelets were lysed and pre-cleared three times for 30 min at 4 °C using 100 µl of protein G-Sepharose (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). The pre-cleared lysates were then incubated with 10 µg of anti-P-selectin mAbs and 10 µl of protein G-Sepharose beads, and the immunoprecipitates were washed and analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the proteins were transferred to an Immobilon-P membrane and probed with streptavidin–horseradish peroxidase (SA-HRP, Sigma-Aldrich). After 60 min, the membrane was developed using ECL Western blotting detection reagents (PerkinElmer Life Sciences, Boston, MA, USA).

2.3. Purification of rat P-selectin

Anti-rat P-selectin mAb A116, purified by Protein G-Sepharose, was coupled to 1 ml HiTrap NHS-activated-Sepharose (GE Healthcare Bio-Sciences Corp.) according to the manufacturer's instructions. The thrombin-activated rat platelets were lysed with 1% NP-40 in PBS. The sample was spun at 100,000×g for 30 min, and the supernatant was pre-cleared with protein G-Sepharose. The supernatant was then applied to the mAb A116-Sepharose column at 4 °C. After washing the column, the bound fraction was eluted with 0.1 M glycine–HCl, pH 2.7, and neutralized immediately after elution. The bound rat P-selectin fraction was separated by SDS-PAGE and visualized by silver staining.

2.4. Lung injury model

Specific pathogen-free, adult (5–6 weeks old), male Wistar, Sprague–Dawley, and Long Evans rats were used. Twenty units of CVF (Sigma-Aldrich)/kg body weight, or 3 mg LPS (Sigma-Aldrich)/kg body weight diluted in saline, or saline alone was injected intravenously (i.v.) as a bolus infusion [15]. At 30 min after the administration of CVF, or 4, 24, 48, or 72 h after the administration of LPS, whole blood was obtained from each rat and mixed with sodium citrate. At the same time, rat lungs were also collected and stored at –80 °C until use. The protocols used for all the animal experiments in this study were approved by the Animal Research Committee of Osaka University.

2.5. Preparation of the sensor chip for BIAcore analysis

The BIAcore system and reagents for interaction analysis were obtained from BIAcore AB, Uppsala, Sweden. Rat P-selectin derived from platelets was immobilized on the sensor chip via primary amine groups according to standard procedures [16,17]. In brief, the immobilization run was performed in HBS, pH 7.4, (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.05% surfactant P20). The carboxylated matrix of the sensor chip was activated with 30 µl of an EDC/NHS (*N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide hydrochloride/*N*-hydroxysuccinimide) mixture. Immobilization of rat P-selectin was performed with 70 µl of 10 µg/ml P-selectin in 100 mM acetate buffer, pH 4.5, and injected at a flow rate of 10 µl/min. Unreacted groups were blocked by injecting 70 µl of ethanolamine–HCl, pH 8.5, at 10 µl/min.

2.6. Epitope mapping using BIAcore

Binding of the anti-rat P-selectin mAbs to the rat P-selectin-immobilized sensor chip described above was detected at a constant flow rate of 10 µl/ml of PBS containing 0.05% Tween-20 in the BIAcore system. A 30-µl aliquot of primary mAb (diluted to 200 µg/ml in PBS containing 0.05% Tween-20) was injected over the rat P-selectin surface. The secondary mAb was then injected in sequence. The sensor chip surface was regenerated with 10 mM glycine–HCl, pH 2.2 (15 µl).

2.7. Sandwich ELISA

To detect soluble rat P-selectin, a sandwich ELISA using two anti-rat P-selectin mAbs or a combination of one mAb and the anti-rat P-selectin polyclonal antibody were performed. Either one anti-rat P-selectin mAb or the anti-rat P-selectin polyclonal antibody was diluted to 10 µg/ml in PBS and incubated for 2 h in each well of a 96-well microtiter plate (Sumilon MS-8596F; Sumitomo Bakelite Co., Tokyo, Japan) at room temperature (RT). The wells were washed with PBS and blocked with 3% BSA in PBS (3% BSA-PBS) overnight at 4 °C. The wells were then washed with PBS containing 0.1% BSA and 0.05% Tween-20 (BT-PBS) and incubated with 3, 1, 0.3, 0.1, 0.03, 0.01 or 0 µg/ml rat P-selectin-IgG, rat P-selectin derived from platelets, or rat plasma P-selectin diluted with PBS containing 0.1% BSA, 0.05% Tween-20, and 10 mM EDTA, for 2 h at RT. The wells were washed with BT-PBS, 2 µg/ml biotin-conjugated anti-rat P-selectin mAb was added, and the plates were incubated for 1 h at RT. The plates were washed with BT-PBS, and then peroxidase-conjugated streptavidin (1:500, Zymed Laboratories, Inc., South San Francisco, CA, USA) was added and the plates were incubated for 60 min at RT. After another wash with BT-PBS, 50 µl per well of *o*-phenylenediamine (0.4 mg/ml) in 50 mM sodium citrate, 100 mM Na₂HPO₄, pH 5.6 containing 0.012% H₂O₂ was added, and the chromogenic reaction was processed for 2 min at room temperature. After the reaction, 8 N H₂SO₄ was added to each well, and the plate was read at 490 nm in a microtiter plate reader (InterMed Co., Tokyo, Japan).

2.8. Tissue myeloperoxidase activity

Myeloperoxidase (MPO) activity was measured to assess the influx of neutrophils into the lung. Lysates from casein-elicited rat peritoneal neutrophils were used as a control. Lung samples were homogenized using 2 ml homogenization buffer (PBS containing 1 mM phenylmethanesulfonyl fluoride, 7.2 µM pepstatin A, 10 µM leupeptin, and 1% NP-40) and incubated for 4 h at 4 °C. The mixture was then spun for 20 min at 25,000×g to remove insoluble materials. The protein concentration of the lysates was adjusted, and the MPO activity was assayed by measuring the change in absorbance (490 nm) using H₂O₂ and *o*-phenylenediamine as described above.

2.9. Bronchoalveolar Lavage (BAL)

BAL was performed with 10 ml cold PBS. The BAL fluid was centrifuged and the supernatant aliquoted and stored at –80 °C until further analysis. Levels of TNF (tumor necrosis factor)-α and IL (interleukin)-1β in the BAL fluid were determined using an ELISA kit (R&D Systems Europe, Ltd., Abingdon, UK). Spectrophotometric measurement of samples and standards were carried out in duplicate using a microplate reader (Bio-Rad Lab., Hercules, CA, USA). Results were recorded as optical densities, plotted against the linear portion of the standard curve, and expressed as picograms cytokine per ml of BAL fluid.

2.10. Lung histology

Histopathologic changes induced by LPS were evaluated in three rats. Briefly, saline-treated rats or LPS-treated rats were prepared as described above. The lungs were gently infiltrated with 1% low melting point agarose (Nacalai Tesque, Inc., Kyoto, Japan) at 42 °C through the trachea. Lungs were then removed *en bloc*, and fixed in 4% paraformaldehyde, 0.23 M sucrose solution overnight. The sample was then incubated in 20% sucrose/PBS for 12 h, and then

in 30% sucrose/PBS for another 12 h. The tissue was embedded, and the 7- μ m sections were stained with hematoxylin–eosin (Wako Chemicals, Osaka, Japan).

2.11. Statistical analysis

Student's *t*-test was used for statistical analysis. The correlations between P-selectin concentration and MPO activity were examined using Pearson's product moment correlation coefficient.

3. Results

3.1. Immunoprecipitation and purification of rat P-selectin

We previously established nine hybridoma cell lines secreting anti-rat P-selectin mAb [14]. To develop an ELISA system, it was necessary to determine if the antibodies could immunoprecipitate rat P-selectin. Three of the anti-rat P-selectin antibodies, A109, A116, and A216, specifically immunoprecipitated a protein appearing as a single band of approximately 130 kDa, which corresponds to the molecular size of rat P-selectin, from thrombin-activated rat platelets (Fig. 1A). The anti-rat P-selectin mAb C215 immunoprecipitated the same protein, but its affinity for rat P-selectin seemed lower than that of mAbs A109, A116, or A216; only a faint band could be detected with mAb C215. Based on these results, we prepared a HiTrap NHS-activated Sepharose column coupled with mAb A116, and used it to purify rat P-selectin from lysates of thrombin-stimulated platelets. This rat P-selectin had a purity of more than 95% (Fig. 1B) and a recovery of about 10 μ g/rat.

3.2. Classification of anti-rat P-selectin mAbs into 5 epitope groups

Real-time biospecific interaction analysis (BIAcore), which is based on changes in surface plasmon resonance, was used to classify the epitopes of the nine anti-rat P-selectin mAbs [14]. Examples of sensorgrams for assessing the antibody competition are shown in Fig. 2A. Sensorgram c indicates that the two antibodies tested had distinct epitopes, whereas sensorgram d indicates that the two antibodies tested had overlapping epitopes. The reactivity patterns of the mAbs determined as described above are shown in a 9 \times 9 matrix table (Fig. 2B). For example, the epitopes of mAbs C215 and A109 were different, since pre-occupation of the P-selectin-immobilized sensor chip with mAb C215 failed to affect the binding of mAb A109, and *vice versa*. In contrast, the epitopes of mAbs 136 and 137 were overlapping, since each of these mAbs interfered with the ability of the other to bind its epitope on the sensor chip. Based on these results, the nine anti-rat P-selectin mAbs were classified into five groups as follows: group 1 (mAbs 118, 136, 137), group 2 (mAbs A109, A116, A216), group 3 (mAb C215), group 4 (mAb A123), and group 5 (mAb C104).

3.3. Detection of soluble rat P-selectin by ELISA

P-selectin is rapidly expressed on activated platelets and endothelial cells and then shed from the cell surface or taken up by endocytosis [18,19]. We hypothesized that circulating soluble P-selectin might be elevated during inflammation and reflect the extent of lung injury. We first attempted to develop a sandwich ELISA using two mAbs that efficiently immunoprecipitated soluble rat P-selectin, but we could not detect P-selectin using any combinations of mAbs A109, A116, and A216 (data not shown), probably because these mAbs fall into the same epitope group. We therefore performed sandwich ELISAs using combinations of the anti-rat P-selectin polyclonal antibody and one of the nine anti-rat P-selectin mAbs (Fig. 3A). Rat P-selectin derived from platelets or rat P-selectin-IgG was added to wells coated with the anti-rat P-selectin polyclonal antibody. The wells were then incubated with biotinylated anti-rat P-selectin mAb A118, 136, 137, A109, A116, A123, A216, C104, or C215. When the biotinylated A109, A116, or A216 mAb was used, native rat P-selectin derived from platelets was detected. However, when the other biotinylated mAbs were used, only recombinant rat P-selectin-IgG but not native rat P-selectin was detected. In the ELISA performed with the anti-rat P-selectin polyclonal antibody and biotinylated A116 mAb, the detection limit was about 80 ng/ml P-selectin (Fig. 3B).

3.4. Circulating soluble P-selectin levels increase in acute lung injury

We next measured the levels of soluble P-selectin *in vivo* using the CVF model. In this model, lung injury is caused by a massive infiltration of neutrophils, a process in which P-selectin plays a major role. To induce acute lung injury, the CVF was infused into the rats intravenously (i.v.). Lung and plasma

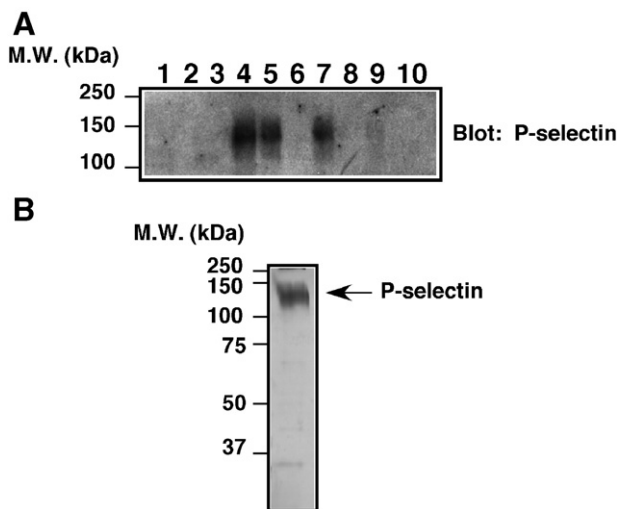


Fig. 1. Immunoprecipitation and purification of rat P-selectin with anti-P-selectin mAb. (A) Immunoprecipitation of P-selectin from rat platelets using mAbs. Rat thrombin-activated platelets were surface-biotinylated and immunoprecipitated with the mAbs (lane 1, 118; lane 2, 136; lane 3, 137; lane 4, A109; lane 5, A116; lane 6, A123; lane 7, A216; lane 8, C104; lane 9, C215) or hamster IgG (lane 10). Samples were run on SDS-PAGE 8% gels under reducing conditions and blotted onto a PVDF membrane. The membrane was treated with SA-HRP and the bands were detected with ECL. (B) Affinity purification of rat P-selectin with an anti-P-selectin mAb A116-Sepharose column. Purified rat P-selectin was run on an SDS-PAGE 8% gel under reducing conditions, and the band was visualized by silver staining.

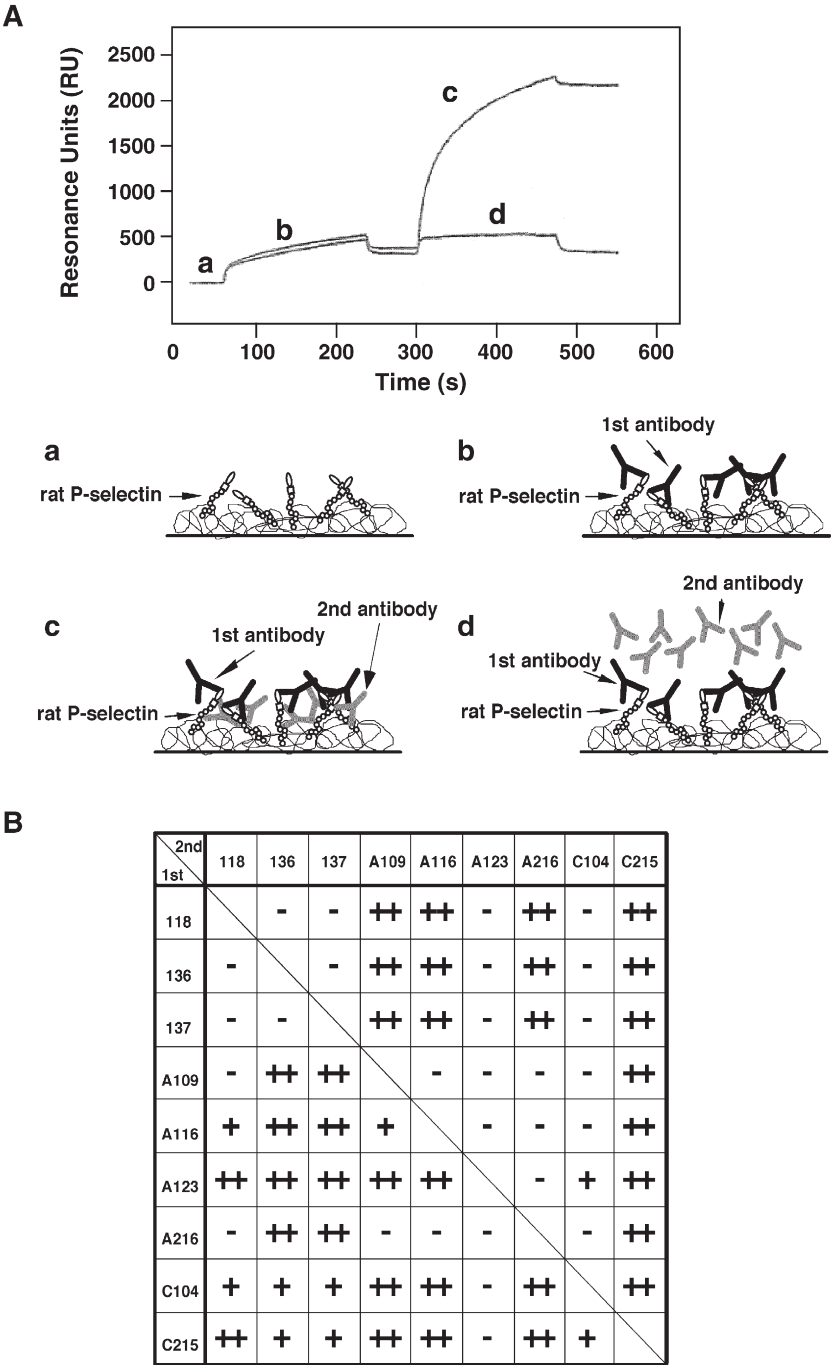


Fig. 2. Epitope groups defined by competitive inhibition studies. (A) Representative sensorgrams obtained from competitive binding experiments by BIAcore. The second mAb was applied to a sensor chip pre-saturated with the first mAb, as described in Materials and methods. When the epitopes recognized by the two mAbs were the same or different, a sensorgram resembling d or c, respectively, was obtained after application of the second antibody. Schematic representations of the binding of mAbs to the sensor chip at various points (a to d) are shown below the sensorgram. (B) Reactivity pattern matrix obtained from a competitive binding experiment by BIAcore. The resonance units obtained after the second mAb application minus those after the first mAb application were calculated and are shown as symbols: >1000 resonance units (++), 300 to 999 resonance units (+), and <300 resonance units (-).

samples were collected 30 min after the i.v. infusion, and the MPO activity and the soluble P-selectin concentration were measured, respectively (Table 1). MPO is a myeloid cell-specific peroxidase, and its activity reflects the neutrophil content in the lung. As shown in Table 1, after the i.v. infusion of CVF, the MPO activity increased to a level above the control values obtained after the infusion of saline, indicating that neutrophil

infiltration was induced by the CVF infusion. Table 1 also shows that the circulating P-selectin values in Long Evens rats was about six times higher than those in the other rat strains in this acute lung injury model. Importantly, the soluble P-selectin levels detected by the sandwich ELISA correlated with the level of MPO activity in the lung, indicating that soluble P-selectin levels reflect the severity of the inflammation in the lung.

We also examined another lung injury model, which uses LPS. After i.v. infusion of LPS, plasma and lung samples were collected at 4, 24, 48, and 72 h, and the soluble P-selectin concentration and MPO activity were measured. As shown in Fig. 4A and B, the circulating P-selectin in the SD rats was elevated to two- to three-fold higher than in Wistar rats, and this elevation correlated with the MPO activity. The coefficients of correlation between P-selectin concentration and MPO activity for SD and Wistar rats (from 0 to 24 h) were $r=0.996$ and $r=0.859$, respectively (Fig. 4C). Hematoxylin–eosin staining of the lung tissues indicated that a large number of leukocytes accumulated in the parenchyma as well as in the alveolar space of the LPS-treated Wistar rats, and, to a larger extent, in the same sites of the LPS-treated SD rats (Fig. 4D to G). As shown in Table 2, total protein concentration in the BAL fluid 4 h after LPS administration in SD rats was higher than that in Wistar rats, indicating that permeability of lung epithelial barrier increased more significantly in SD rats than in Wistar rats. In

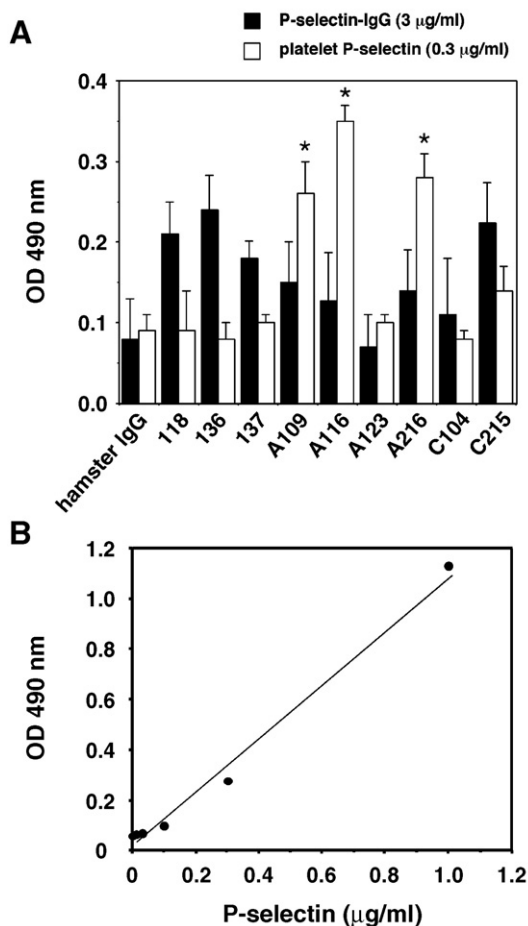


Fig. 3. Detection of the soluble form of rat P-selectin by sandwich ELISA. (A) Rat P-selectin-IgG or rat platelet P-selectin was added to anti-rat P-selectin polyclonal antibody-coated wells. The captured antigen was detected with biotinylated 118, 136, 137, A109, A116, A123, A216, C104, and C215 mAbs. Data are expressed as means \pm SD of triplicate determinations. *, $P<0.05$, versus hamster IgG control. (B) Standard curve of rat platelet P-selectin by sandwich ELISA using a combination of anti-rat P-selectin polyclonal antibody and biotinylated A116 mAb. Coefficient of correlation, $r=0.993$.

Table 1

Elevation of plasma P-selectin levels in CVF-treated rats

	P-selectin level		MPO activity
	(OD 490 nm) ^a	(ng/ml) ^b	(OD 490 nm) ^c
Normal Wistar rat 1	0.081 \pm 0.009	n.d. ^d	0.013 \pm 0.003
Normal Wistar rat 2	0.080 \pm 0.005	n.d.	0.103 \pm 0.005
Normal Wistar rat 3	0.068 \pm 0.005	n.d.	0.121 \pm 0.005
Normal SD rat 1	0.075 \pm 0.004	n.d.	0.125 \pm 0.005
Normal SD rat 2	0.091 \pm 0.003	n.d.	0.075 \pm 0.003
Normal Long Evans rat 1	0.070 \pm 0.007	n.d.	0.043 \pm 0.005
Normal Long Evans rat 2	0.072 \pm 0.004	n.d.	0.065 \pm 0.003
Saline Wistar rat 1	0.071 \pm 0.007	n.d.	0.163 \pm 0.005
Saline Wistar rat 2	0.071 \pm 0.006	n.d.	0.195 \pm 0.003
Saline Wistar rat 3	0.067 \pm 0.008	n.d.	0.113 \pm 0.005
Saline Wistar rat 4	0.067 \pm 0.003	n.d.	0.120 \pm 0.005
Saline SD rat 1	0.077 \pm 0.005	n.d.	0.162 \pm 0.004
Saline SD rat 2	0.069 \pm 0.003	n.d.	0.155 \pm 0.005
Saline SD rat 3	0.081 \pm 0.003	n.d.	0.192 \pm 0.003
Saline Long Evans rat 1	0.087 \pm 0.009	n.d.	0.183 \pm 0.005
Saline Long Evans rat 2	0.057 \pm 0.005	n.d.	0.173 \pm 0.005
CVF Wistar rat 1	0.155 \pm 0.021	110	0.583 \pm 0.014
CVF Wistar rat 2	0.132 \pm 0.015	100	0.326 \pm 0.052
CVF Wistar rat 3	0.121 \pm 0.013	90	0.412 \pm 0.032
CVF SD rat 1	0.221 \pm 0.017	220	0.529 \pm 0.047
CVF SD rat 2	0.184 \pm 0.023	190	0.519 \pm 0.012
CVF SD rat 3	0.193 \pm 0.018	200	0.413 \pm 0.038
CVF Long Evans rat 1	0.895 \pm 0.006	850	0.883 \pm 0.034
CVF Long Evans rat 2	0.822 \pm 0.020	790	0.792 \pm 0.072
CVF Long Evans rat 3	0.781 \pm 0.011	700	0.724 \pm 0.067

^a OD 490 nm was determined by the sandwich ELISA using the anti-P-selectin polyclonal antibody and biotinylated A116 as described in Materials and methods.

^b Plasma P-selectin concentration (ng/ml) was determined by the standard curve shown in Fig. 3B.

^c MPO activity (OD 490 nm) in the lung was determined as described in Materials and methods.

^d n.d.: Not detectable.

addition, concentrations of TNF- α and IL-1 β , proinflammatory cytokines, in the BAL fluid, also increased more significantly in SD rats.

4. Discussion

We have developed an ELISA system to measure soluble P-selectin in the rat. We previously established nine anti-rat P-selectin mAbs that bind to thrombin-stimulated platelets [14]. Among these mAbs, A109, A116, A216, and C215 could immunoprecipitate P-selectin from platelets, and the others could not (Fig. 1 and Table 1). The A109, A116, and A216 mAbs also worked well in an ELISA for detecting native P-selectin from platelets, and the other mAbs did not, although they could detect recombinant P-selectin-IgG chimeric protein (Fig. 3). These results are probably attributable to differences in the immunogens. A109, A116, A123, and A216 were established by immunizing hamsters with activated platelets prepared from rats, and the other mAbs were raised against recombinant P-selectin-IgG chimeric protein [14]. Native P-selectin consists of a lectin, an epidermal growth factor-like domain, and nine short consensus repeats; the recombinant P-selectin-IgG protein lacks eight of the short consensus repeats.

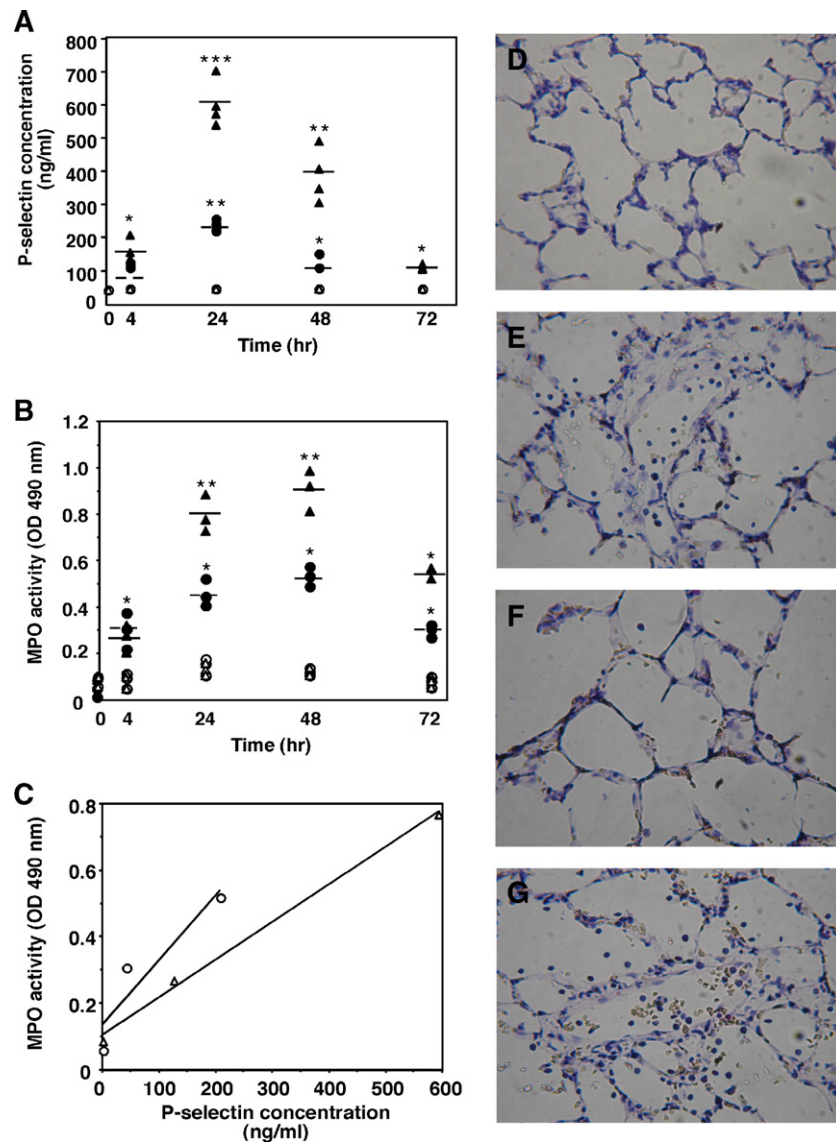


Fig. 4. Time dependency of the plasma P-selectin levels and MPO activities in the LPS-induced rat lung injury model. Plasma and lung lysates were prepared from LPS-(closed triangles, SD rats; closed circles, Wistar rats) or saline-(open triangles, SD rats; open circles, Wistar rats) treated rats at the indicated time points, and the soluble P-selectin concentration (A) and MPO activity (B), respectively, were measured as described in Materials and methods. The horizontal bar indicates the mean value for each group (solid lines, LPS-treated SD rats; dotted line, LPS-treated Wistar rats). * $P < 0.05$, ** $P < 0.005$, and *** $P < 0.001$, versus saline-treated mice. (C) Correlation between P-selectin concentration and MPO activity (from zero to 24 h). The coefficients of correlation between the two parameters for SD and Wistar rats were $r = 0.996$ and $r = 0.859$, respectively. (D–G) Lung histology. Lungs were obtained from Wistar (D and E) or SD (F and G) rats 4 h after the administration of saline (D and F) or LPS (E and G). The tissue sections were stained with hematoxylin–eosin as described in Materials and methods.

The lack of these domains in the recombinant P-selectin-IgG protein and its fusion with the Fc region of IgG may generate steric distortions that affect its immunogenicity.

The ELISA reported here is sensitive enough to quantify physiological concentrations of plasma P-selectin after the induction of inflammation (Table 1 and Fig. 4). Although Misugi et al. [20] developed a non-sandwich ELISA to detect rat soluble P-selectin, their assay might not have been accurate enough to measure the physiological concentration of soluble P-selectin in plasma, because it used only anti-human P-selectin polyclonal antibody. In contrast, our sandwich ELISA system allows the accurate measurement of soluble P-selectin in plasma.

Using the newly developed ELISA, we determined the plasma P-selectin levels during acute lung injury in the rat. The soluble P-selectin levels correlated well with the extent of inflammation, suggesting that measuring P-selectin levels in plasma could be clinically informative. The level of soluble P-selectin in plasma increased in all the rat strains examined, although the amount differed among the strains. In the CVF-injected Long Evans rats, the plasma P-selectin level increased to approximately 800 ng/ml 30 min after injection (Table 1). The increase in plasma P-selectin in Wistar and SD rats was also detectable using our ELISA, although the increase was smaller than in the Long Evans rats. In the LPS-injected SD rats, the plasma P-selectin level increased 4 h after the injection, and

Table 2
Total protein and cytokines in BAL fluid collected from the lungs after challenge with LPS^a

	Wistar rat		SD rat	
	Saline	LPS	Saline	LPS
Protein (mg/ml)	0.21±0.02	1.18±0.52	0.50±0.03	2.85±0.68
TNF-α (pg/ml)	912±29.4	2680±201	798±141	6900±189
IL-1β (pg/ml)	ND ^b	440±16.9	ND ^b	730±16.3

Data are the mean±SEM of three experiments for each parameter.

^a Lungs from rats were obtained 4 h after the administration of saline or LPS. Total protein was measured by ultra-violet spectrometry. TNF-α and IL-1β levels in the lungs were measured by ELISA.

^b ND, not detectable.

reached approximately 600 ng/ml by 24 h after the injection, which was higher than that observed in Wistar rats (Fig. 4A). Considering the previous report that upregulation of P-selectin in the inflamed sites reached a maximum between 4 and 8 h after LPS administration [21] and our finding that the soluble P-selectin levels gradually increased thereafter reaching a maximum at 24 h after LPS administration, it is most likely that soluble P-selectin in plasma is derived from the sites of inflammation. Consistent with the notions above, histological examination indicated that relatively a larger number of leukocyte extravasation was observed in SD rats than in Wistar rats 4 h after LPS administration (Fig. 4D to G). In addition, only 25 out of 52 SD rats survived 24 h after LPS administration, whereas 24 out of 24 Wistar rats survived at the same time point after LPS administration (unpublished observation). In accordance with these findings, total protein and proinflammatory cytokine concentrations in the BAL fluids increased more significantly in SD rats than in Wistar rats (Table 2). The differences in the increase of plasma P-selectin levels and P-selectin-dependent lung injury may be owing to differences in the genetic backgrounds of these rats.

In normal, healthy humans, the plasma contains low levels of soluble P-selectin (30–60 ng/ml) [22]. In patients with vascular disorders such as hypertension, [23] hypercholesterol anemia [24], and unstable angina [25], levels of soluble P-selectin ranging from 120 to 200 ng/ml, with extreme values up to 1000 ng/ml, have been reported. Soluble P-selectin may also serve as a clinical biomarker of these diseases.

Aside from the possible clinical usefulness of this finding, however, the functional role of soluble P-selectin *per se* is not clear. In the CVF model of lung injury, rat lung inflammation is inhibited by treatment with soluble P-selectin-IgG [26] or anti-P-selectin mAb [27]. In addition, in the LPS model of lung injury, rat lung inflammation is inhibited by treatment with soluble P-selectin [28] or an anti-P-selectin mAb [29], suggesting that soluble P-selectin might have some regulatory function exerted by inhibiting the interaction between P-selectin and its ligand. It is well known that P-selectin binds to P-selectin glycoprotein ligand-1 (PSGL-1) expressed on the surface of neutrophils. We previously reported that P-selectin also interacts with glycosaminoglycans that have specific structures [30,31]. Since glycosaminoglycans are implicated in certain lung injuries [32,33] and their biosynthesis is up-regulated during lung injury

[34], glycosaminoglycans may also contribute to P-selectin-mediated acute lung injuries. In the inflammatory setting, soluble P-selectin secreted into the plasma might therefore function in the regulation of inflammatory reactions by disrupting the binding of P-selectin to PSGL-1 and glycosaminoglycans.

In summary, we have developed a sandwich ELISA that enables us to measure accurately the amount of soluble rat P-selectin in plasma. By applying this system to rat lung inflammatory models, we found that these P-selectin levels were well correlated with the extent of lung injury. Our results demonstrate that soluble P-selectin levels reflect lung inflammation, which is of clinical importance for detecting and preventing severe lung injury.

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